Proceedings
OF THE
National Academy of Sciences
OF THE UNITED STATES OF AMERICA

November 1986 | Volume 83, Number 21 | pp. 8019–8434

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Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8137–8141, November 1986
Biochemistry

Linking functional domains of the human insulin receptor with the bacterial aspartate receptor

(chimeric receptors/transmembrane signaling/protein folding)

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Contributed by Daniel E. Koshland, Jr., July 31, 1986

ABSTRACT **A hybrid receptor has been constructed that is composed of the extracellular domain of the human insulin receptor fused to the transmembrane and cytoplasmic domains of the bacterial aspartate chemoreceptor. This hybrid protein can be expressed in rodent (CHO) cells and displays several functional features comparable to wild-type insulin receptor. It is localized to the cell surface, binds insulin with high affinity, forms oligomers, and is recognized by conformation-specific monoclonal antibodies. Although most of the expressed protein accumulates as a 180-kDa proreceptor, some processed 135-kDa receptor can be detected on the cell surface by covalent cross-linking. Expression of the hybrid receptor inhibits the insulin-activated uptake of 2-deoxyglucose by CHO cells. Thus, this hybrid is partially functional and can be processed; however, it is incapable of native transmembrane signaling. The results indicate that the intact domains of different types of receptors can retain some of the native features in a hybrid molecule but specific requirements will need to be satisfied for transmembrane signaling.**

Signal transduction via membrane receptors involves discrete external and cytoplasmic domains with coordinated input and output functions. Receptors that appear to have a single or very few membrane spanning regions are an important class for study. Representative of this structure are the epidermal growth factor receptor (1), the insulin receptor (2, 3), the low density lipoprotein receptors (4), and the aspartate and serine receptors of bacterial chemotaxis (5, 6). The receptor has a number of functions: to recognize a specific stimulus, to communicate information across the membrane, and to generate a signal that then is processed in the interior of the cell. To clarify the manner in which these processes are carried out, we have fused domains from two apparently unrelated receptors, the human insulin receptor (IR) and the bacterial aspartate receptor (AR), and analyzed the properties of the hybrid protein (IAR).

The native IR is a disulfide-linked heterotetramer composed of two α and two β subunits (7). Insulin binds to this receptor with high affinity and stimulates autophosphorylation of the β subunit on tyrosine residues. This tyrosine phosphokinase activity is characteristic of a class of transforming proteins encoded by oncogenes, including $pp60^{src}$ and the receptors for platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor 1 (8). The primary sequence of the IR predicts a single polypeptide chain precursor encoding both the α and β subunits that is proteolytically processed during biosynthesis to generate α and β subunits (2, 3). Consistent with prior biochemical studies, the sequence indicates that it is comprised of an extracellular domain containing all of the α and one-third of the β subunit, a stretch of hydrophobic amino acids that is presumed to span the membrane, and an intracellular domain that is the remaining two-thirds of the β subunit.

The AR of chemotaxis is likewise composed of an extracellular domain that binds aspartate with high affinity and specificity (6). It contains two transmembrane segments, one near the N terminus and the other near the middle of the molecule, and a C-terminal cytoplasmic portion that generates the signal that controls flagella function. The cytoplasmic domain also becomes methylated at four distinct glutamate residues (9) that have been identified with the adaptation properties of the receptor (10, 11).

The domains of the two widely different functional receptors were connected to form a chimeric protein by recombinant DNA methods. The external N-terminal portion of the IR was split near residue 921 in the β chain and attached to the C-terminal portion beginning with residue 171 of the AR; a new glycine residue is introduced at the junction. Other combinations of the IR and AR have been synthesized, and their properties will be reported subsequently.

MATERIALS AND METHODS

Construction of the IAR. The hybrid IAR cDNA was assembled in plasmid pECE, a 2.9-kilobase (kb) expression vector (12) with the following features: (*i*) a simian virus 40 early promoter for expression in mammalian cells, (*ii*) a polylinker that includes 5′ *Hin*dIII and 3′ *Xba* I sites not present in either the IR cDNA (2) or AR gene (6), and (*iii*) 3′ sequences containing the polyadenylylation signal and poly(A) tract of the simian virus 40 early genes, as the bacterial AR gene (the 3′ end of the hybrid IAR) does not have them. All manipulations of these DNAs were according to standard methods (13). Enzymes were from New England Biolabs or Boehringer Mannheim.

Plasmid peT, which contains the full-length (≈4.5-kb) IR cDNA cloned into the *Eco*RI–*Xba* I sites of pECE (12), was (*i*) digested partially with *Aat* II (which leaves a 3′ overhang); (*ii*) rendered blunt-ended by treatment with the large fragment of *Escherichia coli* DNA polymerase I (Klenow) for 30 min at room temperature, followed by an additional 30 min at room temperature in the presence of all four deoxynucleotide triphosphates (P-L Biochemicals; 100 μM); and (*iii*) digested with *Xba* I. The ≈5.8-kb fragment containing the pECE vector and 2983 base pairs of the IR cDNA, with 5′ blunt and

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Abbreviations: IR, insulin receptor; AR, aspartate receptor; IAR, hybrid IR-AR protein; kb, kilobase(s); FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody.

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3′ *Xba* I ends, was purified by electrophoresis on low-gelling temperature agarose (SeaPlaque; FMC, Rockland, ME).

The AR gene fragment used in this construction was derived from pRK41, which contains the AR gene as a 2.35-kb *Cla* I fragment cloned into the unique *Cla* I site of pBR322 (6). The AR gene fragment was provided with a 3′ *Xba* I site as follows: (*i*) pRK41 was digested to completion with *Sma* I (base pair 744 of AR gene) and *Eco*RI (which does not cut within AR, but cuts to the 3′ side of the gene at the *Eco*RI site of pBR322; (*ii*) the ≈1.6-kb *Sma* I (5′)–*Eco*RI (3′) fragment was purified by electrophoresis on SeaPlaque agarose and ligated with *Sma* I–*Eco*RI-digested pECE; and (*iii*) this AR fragment was excised from this intermediate plasmid by digestion with *Sma* I and *Xba* I, and purified on SeaPlaque agarose. This fragment was ligated with the above ≈5.8-kb *Aat* II blunt-end–*Xba* I fragment containing pECE and the IR sequences. The resulting plasmid, designated peIAR.1, was used in the present experiments for the expression of the IAR hybrid protein in CHO cells as described (12, 14) (see also text). The expected sequence at the IR/AR junction of this construction is shown in scheme 1.

IR residue 921	AR residue 171
Tyr–Leu–Gly–Val–Ser–Glu–Asn	
5′ TAT TTA GGG GTG AGC GAA AAC 3′	

Scheme 1

Here the first guanosine of the glycine codon is contributed by the Asp-922 codon of the IR cDNA [which is interrupted by the *Aat* II site of base pair 2983 (2)] and the second and third guanosines of the glycine codon are contributed by the Arg-170 codon of the AR [which is interrupted by the *Sma* I site at base pair 744 of the AR gene (6)].

This DNA sequence was confirmed by subcloning a fragment (5′ *Pst* I site of bp 2868 of IR cDNA, 3′ *Xba* I site) of the hybrid cDNA containing this junction into M13-mp18 (15) and determining its sequence by the dideoxy-chain terminator method (16). The IAR hybrid protein thus contains 921 residues derived from the IR [all of the α subunit and all of the extracellular portion of the β subunit, except 8 amino acids prior to the transmembrane domain (2)]. A new glycine residue is created at the hybrid junction, which connects to 18 residues of the extracellular (periplasmic) domain of the aspartate receptor, the transmembrane domain (24 amino acids), and entire cytoplasmic domain (341 amino acids) of the bacterial protein (6).

peIAR.1 DNA was introduced into wild-type Chinese hamster ovary (CHO) cells as a calcium phosphate precipitate with pSV2neo DNA as described (12, 14). Primary transformants were selected by the use of the antibiotic G418 (GIBCO).

Total cellular RNA dot blots prepared from primary transformants were screened for the expression of the expected hybrid mRNA with an AR DNA probe (data not shown). One such transformant, CHO.IAR-4, which expressed the highest level of AR-related mRNA, was selected for further analysis.

Fluorescence-activated cell sorting (FACS), ^{125}I-labeled insulin binding, purification of the hybrid IAR protein, metabolic labeling of cells, immunoprecipitations, and uptake of 2-deoxy[^{3}H]glucose were performed as described (12). Covalent cross-linking of ^{125}I-labeled insulin to intact cells was as described in the legend to Fig. 4.

RESULTS

To assess whether the IAR protein is expressed on the surface of the transfected cells, we employed indirect immunofluorescence and FACS (12), using a monoclonal antibody (mAb) specific for the IR. Since the antibody blocks insulin binding to cells and only recognizes the native IR protein, it provides an assay for the native conformation of the extracellular domain of the IR (17). Initial analysis of the CHO.IAR-4 cells (Chinese hamster ovary cells containing IAR-4) by FACS revealed a population (≈10% of the total) that had a fluorescent intensity 10-fold brighter than the controls. We enriched these cells by aseptically sorting the most intense cells (≈2.5% of the total). These cells comprised a broad peak that was 7 times more fluorescent than the controls. Pure cell lines were generated from this population through subcloning by limiting dilution. One such cell line, hereafter referred to as IAR cells, exhibited a level of fluorescence approximately 2.5 times the control level, consistent with $\approx 2 \times 10^4$ receptors per cell (Fig. 1; cf. ref. 12). These results with the IR mAb suggest that the extracellular domain of the IAR, which is anchored to the cell surface by the transmembrane domain of the bacterial receptor, has assumed a wild-type conformation. Furthermore, this IAR has a nearly wild-type affinity for insulin (Fig. 2). The concentration of insulin required for half-maximal displacement of ^{125}I-labeled insulin is 1.6 nM for IAR cells as compared to the value 0.8 nM obtained with CHO.T cells, a transfected CHO line that expresses $\approx 5 \times 10^5$ human IRs per cell. A Scatchard analysis of these data indicates $\approx 3 \times 10^4$ high-affinity sites per IAR cell (data not shown), in agreement with the estimation based on FACS.

The biosynthesis of this hybrid receptor in IAR cells was studied by labeling the cells overnight (16 hr) with [^{35}S]methionine. Labeled proteins were examined by NaDodSO$_4$/PAGE following immunoprecipitation with the IR mAb from nonionic detergent extracts. The subunit structure was compared with the wild-type receptor by running parallel lanes of IAR cells and CHO.T cells. The IR mAb specifically recognizes three bands in CHO.T cells, with molecular weights consistent with those of the IR precursor (≈200,000) and the

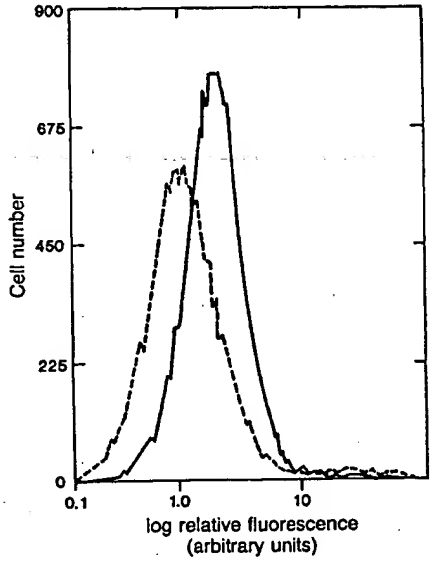

FIG. 1. Analysis of CHO.IAR cells by FACS. A transfected CHO cell line that expresses the IAR hybrid protein (CHO.IAR cells) was generated by the use of FACS as described (see ref. 12 and text). Peak with dashed line: CHO.IAR cells incubated with a control primary antibody (normal mouse IgG; Cappel) and a fluorescein-conjugated secondary antibody (goat anti-mouse IgG; Boehringer Mannheim). Peak with solid line: CHO.IAR cells incubated with a mAb specific for the human IR (17) and a fluorescein-conjugated second antibody.

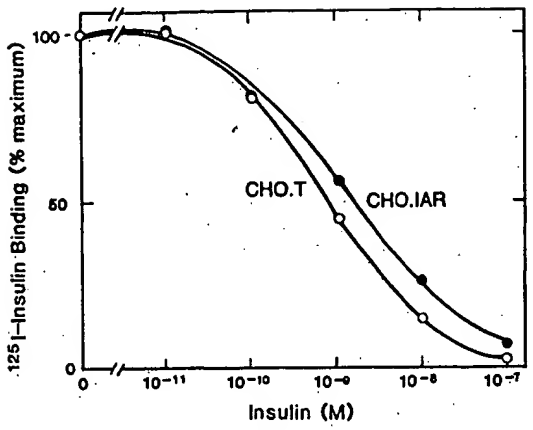

FIG. 2. Binding of ^{125}I-labeled insulin (^{125}I-insulin) to CHO.T cells, which express human IR (12), and CHO.IAR cells. CHO.T (○; 5×10^4 cells per well) or CHO.IAR (●; 5×10^5 cells per well) cells were incubated 16 hr at 4°C with ^{125}I-labeled insulin (30,000 cpm) in the presence of indicated concentrations of unlabeled insulin as described (12). Results are expressed as the percentage of bound ^{125}I-labeled insulin in the absence of unlabeled insulin; 100% values were 3500 cpm (CHO.T) and 2031 cpm (CHO.IAR). Values are an average of two separate experiments with duplicate wells.

α (≈135,000) and β (≈95,000) subunits (Fig. 3, lane 2). These bands are not immunoprecipitated by normal mouse IgG (Fig. 3, lane 1). In contrast, the monoclonal antibody to the human IR recognizes a single band of M_r ≈180,000 in IAR cells (Fig. 3, lane 4), which is absent in the control (Fig. 3, lane 3). The molecular weight of this band is about that expected for the precursor of the hybrid IAR molecule. Furthermore, the ≈180-kDa band can be followed in pulse–chase experiments. After a chase period of 48 hr, the ≈180-kDa labeled band has disappeared, and no processing is observed at intermediate time points.

That this ≈180-kDa molecule also contains AR coding sequences is confirmed by immunoblots of total IAR cellular proteins probed with a rabbit anti-AR antibody. An immunoreactive band of ≈180-kDa is visible on such blots that is not observed in extracts prepared from wild-type CHO cells (data not shown). These results thus confirm that the major (molecular) form of the IAR protein expressed in IAR cells is the ≈180-kDa hybrid proreceptor. The fluorescence assays indicate that there are ≈$2–3 \times 10^4$ cell surface IAR molecules per cell. However, both indirect immunofluorescence of permeabilized IAR cells and net recovery of IAR receptor protein indicate a greater number of intracellular IARs (by a factor of 10–100), suggesting inefficient transport to the cell surface (see below).

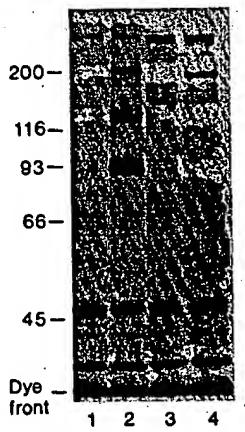

FIG. 3. Subunit structure of human IR and IAR expressed in transfected CHO cells. Semi-confluent 100-mm dishes of CHO.T (lanes 1 and 2) and CHO.IAR (lanes 3 and 4) cells were metabolically labeled with [^{35}S]methionine and immunoprecipitated as described with either normal mouse IgG (lanes 1 and 3) or monoclonal antibody 5D9 (lanes 2 and 4), which blocks insulin binding to the human IR (18). Labeled proteins were reduced and analyzed by NaDodSO$_4$/PAGE and by autoradiography. Molecular sizes (in kDa) of protein standards are indicated.

What is the nature of the IAR protein expressed on the surface of these cells? To address this question, intact IAR cells were covalently cross-linked with ^{125}I-labeled insulin in the absence or presence of 10 μM unlabeled insulin. Labeled proteins of lysed cells were examined by NaDodSO$_4$/PAGE and autoradiography (Fig. 4). Under reducing conditions, a single labeled band of ≈135 kDa is visible in IAR cells incubated with ^{125}I-labeled insulin alone (lane 1), and unlabeled insulin specifically displaced labeled insulin in this band (lane 2). The ≈135-kDa subunit of the endogenous rodent receptor is not visible under these conditions, since only ≈$2–3 \times 10^3$ high-affinity IRs exist per cell (19). Thus, despite the low level of IAR precursor processing observed in metabolic labeling experiments (cf. Fig. 3), the IAR at the cell surface exhibits a mature α subunit. However, the presence of entirely heterologous domains has dramatically reduced the efficient processing of this hybrid IAR receptor.

How does the presence of the heterologous AR domains influence the quaternary structure of the IAR molecule? Electrophoresis under nonreducing conditions of the ^{125}I-labeled insulin cross-linked cell extracts reveals several high molecular weight species (M_r, >>200,000; Fig. 4, lane 3), which are not labeled in the presence of unlabeled insulin (lane 4). This result indicates that the IAR is incorporated into

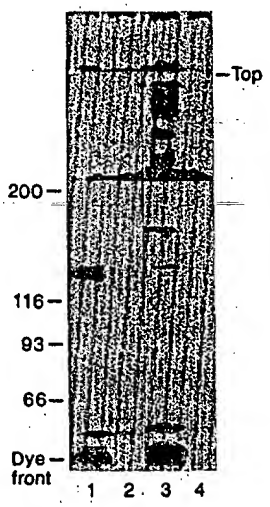

FIG. 4. ^{125}I-insulin cross-linked proteins expressed on the surface of CHO.IAR cells. Confluent CHO.IAR cells in 24-well plates were washed and incubated for 2 hr at 15°C with ^{125}I-labeled insulin (200,000 cpm) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μM insulin. ^{125}I-labeled insulin was then covalently cross-linked to its receptor by the addition of the cross-linking agent disuccinimidyl suberimidate (0.5 mM) for 15 min at 4°C. Reactions were terminated by the addition of 100 mM Tris·HCl, pH 7.5. Cells were then washed, solubilized with 1% Triton X-100, clarified by centrifugation (10,000 × *g*; 10 min, 4°C), and boiled in 1% NaDodSO$_4$ in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 5% (vol/vol) 2-mercaptoethanol. Proteins were analyzed by NaDodSO$_4$/PAGE (5% gel) and by autoradiography of the dried gel. Molecular sizes (in kDa) of protein standards are indicated.

Table 1. 2-Deoxy[^{3}H]glucose uptake by transfected and nontransfected CHO cells

Cell line	IRs per cell, no.	Half-maximal response, insulin [M]	Insulin stimulation, factor
CHO*	2–3 × 10^3	9 × 10^{-10}	3.3
CHO.T†	1.4 × 10^4	3 × 10^{-11}	
CHO.T‡	5 × 10^5	9 × 10^{-12}	
IAR§	2–3 × 10^4	7 × 10^{-9}	2.7

*See ref. 19.
†See ref. 14.
‡See ref. 12.
§Present study.

disulfide-linked oligomers even without the normal cytoplasmic domain. Since the entire AR protein contains no cysteine residues (6), this oligomerization must involve intermolecular disulfide bonds between the IR extracellular domain.

How does the expression of this hybrid IAR influence insulin-activated cell responses? This question can be addressed by monitoring the insulin-activated uptake of 2-deoxy[^{3}H]glucose by the cells, and the data from these experiments are summarized in Table 1. Comparison of the nontransfected CHO cells with IAR cells, displayed in Fig. 5, reveals that the introduced IARs actually decrease the sensitivity of insulin-stimulated uptake by a factor of 10, and also decrease the maximal response by 40%. Thus, the IAR is not merely nonfunctional in transducing an insulin response, but somehow interferes with the response of the endogenous rodent IR as well. Whether this observed deficit occurs by the formation of nonfunctional heterodimers (wild-type rodent IR/mutant IAR), by the inhibition of rodent IR biosynthesis (see ref. 12), or by some other mechanism is not known at present.

That the IAR hybrid does not mediate a response to insulin *in vivo* is not surprising, since there is no IR cytoplasmic domain. However, it is conceivable that this hybrid receptor may be able to transduce a transmembrane signal such as the induced methylation observed in AR. To test this possibility, we reconstituted the IAR protein in an *in vitro* system in which the bacterial AR can be methylated (20). Nonionic detergent extracts (octyl β-D-glucoside) of CHO.IAR cells were incubated in a reaction including bacterial phospholipids, glycerol, purified bacterial methylase, *S*-[^{3}H]adenosylmethionine, and with or without 1 μM porcine insulin. We have thus far been unable to detect receptor methylation in such a system. Similarly, the solubilization of cell membranes by protocols employed for mammalian IRs (17) failed to yield detectable methylation.

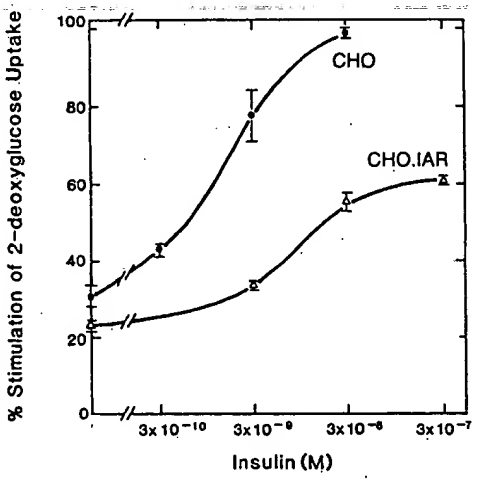

FIG. 5. Insulin stimulation of 2-deoxyglucose uptake. Uptake of 2-deoxy[^{3}H]glucose by wild-type CHO (●) and CHO.IAR (△) cells was measured after a 30-min incubation with indicated concentrations of insulin, as described (12, 14). Values are means ± SD of triplicates and are expressed as a percentage of the maximum CHO response (2860 cpm).

DISCUSSION

The formation of a hybrid receptor containing the external portion of the IR and the cytoplasmic portion of the AR as shown in Fig. 6 has revealed a number of significant features of these receptors. In the first place the external domains of the IR appear to fold into approximately the native conformation, as indicated by insulin binding and a conformation-specific monoclonal antibody. This is surprising, since the transmembrane portion and 8 extracellular amino acids of the IR have been replaced by the transmembrane portion and an extra 19 external amino acids of the AR. Despite these changes the folding of the insulin-binding portion of the receptor is not seriously altered.

Secondly the single transmembrane portion of residues 189–212 of the AR appears to be sufficient to anchor the protein in the membrane. Previous studies have demonstrated that the AR anchors itself in the membrane of bacterial cells, but those receptors contained two transmembrane sequences. Apparently only one of the two is sufficient for anchoring.

The replacement of the mammalian C-terminal domain by the bacterial one apparently does not prevent either expression or processing. This result is consistent with other studies in which C-terminal sequences have proven to be innocuous but also contrasts with studies on proteins in which such domains are critical (21). The processed receptor still gets to the cell surface but it does so with much less efficiency, an indication that the C-terminal domains play a role in the sorting process. Furthermore, it is striking that the IAR actually interferes with the endogenous insulin response of the rodent cells. This may be due to an inhibition of rodent IR biosynthesis or other complex interactions. However, the possibility of mixed oligomers between rodent IR and IAR

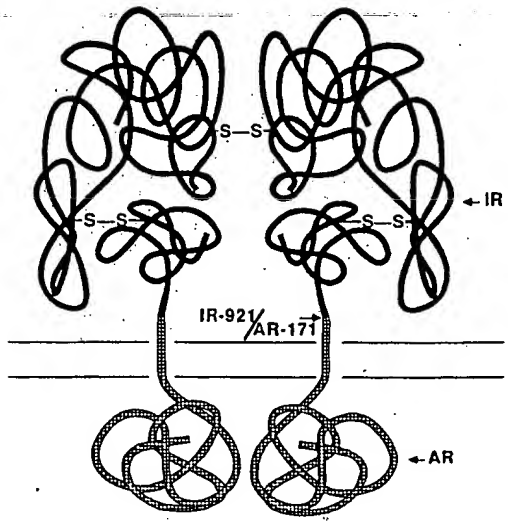

FIG. 6. Schematic model of processed hybrid molecule containing N-terminal portion of IR (heavy black line) and C-terminal portion of AR (cross-hatched line). Horizontal lines indicate membrane region.

subunits is appealing and may hint at the importance of quaternary structure in signal transduction.

The alteration of the sequence also inhibits the transmembrane signaling. Whether the added external amino acids are responsible or whether other factors prevent this transmission must await future experiments.

Chimeric proteins of various types have been made for antibodies (22), receptors (23), enzymes (24), and other proteins. In most of those cases either short signal sequences were substituted or similar domains were interchanged. In analogous studies, truncated receptors have been made in the laboratory (6, 12) or in nature by oncogenic viruses (25) to generate defective proteins. For example, an N-terminal portion of the AR retains aspartate binding properties (26), and proteins with various lengths of the C-terminal portion deleted produce proteins that signal but do not adapt (6). In the case reported here, two very different structural domains have been interchanged. The chance that they would be functional was enhanced by the common structural feature of the single hydrophobic transmembrane sequence. Apparently this similarity was sufficient to retain many of the properties of the individual domains. However, the receptor fusion was sufficiently imperfect to prevent detectable transmembrane signaling. Permutations can now be made on this basic design by assembling the domains at different junctions to reveal the fundamental nature of the signaling mechanism.

We thank our colleagues at the Hormone Research Institute (University of California, San Francisco), the Pharmacology Department (Stanford), and the Biochemistry Department (University of California, Berkeley) for their advice and encouragement during the course of this work. We are grateful to Drs. Lebo and Kan for allowing us access to the Howard Hughes Medical Institute cell sorter and to sorter operators Araxy Bastian and Dr. Paul Dazin. We thank Dr. Joseph Falke and David Sanders for helpful advice on the methylation assays and Bobbi Johnson for help in the tissue culture facilities. We thank Drs. Robert Tjian and Flora Katz for helpful discussions and comments on the manuscript and Ms. Leslie Spector and Ms. Rebecca Boone for assistance in its preparation. We acknowledge the support of the National Institutes of Health and the National Science Foundation. Also we are grateful for fellowships from the following: a Senior Fellowship of the American Cancer Society to L.E.; an Alberta Heritage Foundation Fellowship to D.O.M.; a National Institutes of Health International Fogarty Fellowship to E.C.; a National Science Foundation Fellowship to G.B.; and a Damon Runyon–Walter Winchell Cancer Fund Fellowship to G.R.M.

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